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EX VIVO STEM CELL EXPANSION OF FETAL/ADULT HEPATIC PROGENITOR CELLS AND HEMATOPOIETIC STEM CELLS FOR THE TREATMENT OF LIVER AND PANCREATIC DISEASES			
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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.



No



Yes, the name of the US Government agency and the Government contract number are: _____

Respectfully submitted,

SIGNATURE 

September 17, 2003

Date

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**EX VIVO STEM CELL EXPANSION OF FETAL/ADULT HEPATIC
PROGENITOR CELLS AND HEMATOPOIETIC STEM CELLS FOR THE
TREATMENT OF LIVER AND PANCREATIC DISEASES**

Inventors: Moshe Marikovsky, Tony Peled and Frida Grynspan

Background

Liver transplantation is the only viable option for effectively treating acute liver failure. Currently there are over 14,000 people awaiting liver transplants in the United States. According to the UNOS Scientific Registry 1999 annual report, the median national waiting time in 1998 was 515 days. In addition, HCV is a virus that causes chronic liver disease, cirrhosis and primary liver cancer (HCC) and many patients infected with HCV require liver transplantations. It is estimated that about 4 million people in the United States are infected with hepatitis C, which is about 2% of the population. This makes hepatitis C much more common than HIV infection.

Liver transplantation is severely hampered by the lack of available donors and therefore the search for new treatment modalities among them cell replacement therapy is of outmost importance. One of the strategies is to grow hepatocytes in culture to substitute organ transplantation. Hepatocytes have a great capacity to proliferate *in vivo* but they grow poorly *in vitro*. The proliferation of primary rat hepatocytes *in vitro* have been stimulated by the presence of bone marrow derived stromal cells (Mizuguchi et al, 2001).

Adult stem hepatic cells (oval cells) have also been considered for use as replacement therapy in the treatment of acute liver failure. Oval cells emerge during liver necrosis caused by chemical injury or when hepatocytes are treated with chemicals that block differentiation (Petersen, B.E., 2001). These cells have the potential to differentiate into bile duct epithelium or hepatocytes (Germain et al., 1988) The major obstacle in the use of these cells is the highly restricted number of naturally available oval cells in adult liver, therefore, in order for these cells to represent a viable therapeutic option, expansion techniques need to be developed.

Hematopoietic progenitor cells derived from cord blood, bone marrow or peripheral blood can also be converted into sources of liver or pancreatic cells. Mononuclear UCB cells produced albumin upon cultivation in the presence of hepatic growth factors such as FGF-1, FGF-2, LIF and OSM and had the capacity to develop into functional hepatocytes when transplanted into liver-injured severe combined

immuno-deficient (SCID) mice (Kakinuma et al., 2003). Bone marrow progenitor cells can also develop into active hepatocytes in-vivo under certain circumstances (Petersen et al, 1999; Theise et al., 2000). Cultured BM mononuclear cells in the presence of HGM, Fetal Bovine Serum (FBS), Human Growth Factor (HGF) and Epidermal Growth Factor (EGF) produce hepatocytes-like colonies that are also albumin positive. A subset of these adult rat bone marrow population is positive for hepatocytes growth factor (HGF), receptor c-Met, and α -fetoprotein alongside with hematopoietic stem cell markers such as CD34, Thy-1 and c-kit (Miyazaki et al, 2002). Furthermore, cells expressing albumin, CK18 and HNF (previously referred to as HNF-1, LPB1, and APF) can be generated from primitive, multipotent adult bone marrow-derived progenitor cells. These cells can acquire a hepatocyte phenotype and functional characteristics of hepatocytes (Schwartz et al., 2002).

Embryonic cells can be an alternative source of hepatic cells. Mouse embryonic cells have the potential to differentiate into hepatocytes in vitro in the presence of growth factors for hepatic maturation (Hamazaki et al., 2001). Recently, human Embryonic Stem Cells (hESC) have been shown to differentiate to hepatic cells by the addition of sodium butyrate (Rambhatla et al., 2003). This report suggests that cultivation of hESC or other pluripotent stem cells in large quantities may provide an alternative source for hepatic tissue engraftment.

Type I diabetes mellitus is characterized by a progressive loss of pancreatic beta cells that causes the decrease in insulin production. 17 million people--6.2 percent of the USA population--have diabetes. In 1999, approximately 450,000 deaths occurred among people with diabetes aged 25 years and older. This figure represents about 19 percent of all deaths in the United States in people aged 25 years and older.

The successful replacement of beta cells depends on the availability of human organs supply and the protection of the transplanted cells from immune destruction. Even if immunosuppressive therapy can minimize immune rejection, the shortage of donors is such that it will not be possible to meet the expected demand (Soria et al., 2001). The limited supply of human pancreatic glands, stresses the need to develop alternative approaches to outwit the shortage of cells by searching for new cell sources or expansion technologies.

cells. Genetic manipulation of embryonic stem cells has been performed in mouse embryonic lines that after clonal selection were shown to normalize glycemia in streptozotocin-induced diabetic mice (Soria et al., 2000).

The use of human embryonic stem cells, although promising, encounters numerous practical problems, such as the feeder cell layer that the available lines grow on, immunogenic and tumorogenic issues, and ethical hurdles.

The use of hepatic cells from fetal origin or adult stem hepatic cells (oval cells), are an alternative source for pancreatic-insulin producing cells. Highly purified adult rat hepatic oval cells can trans-differentiate into pancreatic endocrine hormone-producing cells when cultured in a high-glucose environment (Yang et al., 2002). Fetal human progenitor liver cells were induced into insulin-producing cells after expression of the pancreatic duodenal homeobox (PDX1) gene, and their replication capacity enhanced by the additional introduction of the gene of the catalytic subunit of human telomerase (Zalzman et al., 2003).

The successful development of a cell therapy for the treatment of liver and pancreatic diseases based on embryonic, fetal, adult stem cells and/or hematopoietic progenitor cells relies on the availability of sufficient cell number for treatment.

Gamida Cell's aim is to develop expansion techniques that will provide a solution to the limited number of cells obtained during the isolation of specific cell types prior to or after their differentiation or trans-differentiation into hepatic or pancreatic cells. The differentiation can be induced by either various culture conditions or by genetic manipulation of the cells.

Results

1. Gamida Cell expects to develop advanced cell culture methods enabling long-term *ex vivo* expansion of human hepatic or pancreatic progenitor cells derived from fetal or adult tissue.

Preliminary result of hepatocyte cultures derived from mouse adult liver in the presence of EGF, HGF and in the presence of tetraethylpentamine (Tepa) (10 μ M) or in the presence of retinoic acid antagonist (AGN 194310) were obtained (Figure 1). The results demonstrate that Tepa or retinoic acid antagonist induce hepatocytes to proliferate in cell culture for several passages even after trypsin treatment. The cultures treated only with culture media and growth factors revealed fully matured bi-nucleated hepatocytes that stop proliferating after a few passages. Interestingly, treatment of

these cells with Tepa or the antagonist resulted in the appearance of cells that resemble oval cells (stem adult liver cells).

Based on these results the use of Gamida-Cell's technology is expected to increase ex-vivo expansion of fetal liver cells and human adult hepatocytes or oval cells, to obtain sufficient cell numbers in order to successfully treat hepatic and pancreatic diseases

2. Use of Gamida-Cell's technology of ex-vivo expansion of human bone marrow (BM) and peripheral blood (PB) progenitor/stem cells and umbilical cord blood (UCB), is expected to expand also adult hepatocytes, oval cells, fetal liver cells, and cultures of cells exhibiting hepatic-like characteristics (such as hESC or other pluripotent stem cells differentiated into hepatic-like cells). These cells will be induced to differentiate into hepatic or pancreatic progenitor/stem cells as described in "Materials and Methods" to obtain sufficient cell number in order to successfully treat hepatic and pancreatic diseases

3. Induction of differentiation of human BM, PB and UCB progenitor/stem cells, adult hepatocytes, oval cells and fetal liver cells, and multipotent stem cells differentiated into hepatic-like cells into hepatic or pancreatic progenitor/stem cells as described in "Materials and Methods". Use of Gamida-Cell's technology is expected to increase ex vivo expansion of these differentiated hepatic or pancreatic progenitor cells to obtain sufficient cell number in order to successfully treat hepatic and pancreatic diseases..

The resulting expanded cells, the expanded cells induced to differentiate into the hepatic or pancreatic cells, and the cells inducted into hepatic or pancreatic cells followed by their expansion are expected to engraft injured liver and generate functional hepatocyte in SCID mice (NOD/SCID or NOD/SCID/B2M) and/or immuno-deficient rat subjected to one-third partial hepatectomy. Their functionality measured by the presence of human CD45+ cells and their expression of mRNA for human albumin, AFP+ and CK19+. as described in "Materials and Methods. The cell induced to become insulin producing cells are expected to reverse hyperglycemia in 6-week old diabetic NOD-SCID mouse model as previpously described (Zalzman et al., 2003; and Ferber et al.,2003)

Materials and Methods:

Isolation and Culture of adult mouse/rat and human hepatocytes

Hepatocytes are harvested from 3 weeks old VLVC female mice (Harlan Laboratories, Jerusalem, Israel) or Wistar rats and human cells. The livers are extracted and washed twice with DMEM (Beit Haemek, Israel), incubated with DMEM in the presence 0.05% collagenase for 30 minutes at 37 °C, grinded and pass through a 200 μ m mesh. The cells are washed twice, the viable cells counted with trypan blue and plated in 35 mm tissue culture plates coated with collagen. Cells are plated at a density of 4×10^4 /ml in F12 containing 15 mM Hepes, 0.1% glucose, 10 mM sodium bicarbonate, 100units/ml penicillin-streptomycin, glutamine, 0.5-units/ml insulin, 7.5mcg/ml hydrocortisone, and 10% fetal bovine serum, EGF (10ng/ml) and, and HGF (20ng/ml). Medium is changed after 12 hours, the cells washed twice with PBS and new medium added. Medium is changed twice a week.

The cells are also grown in the presence of EGF and, FGF-4 and HGF according to the method of Schwartz et al (2002), and in medium free serum according to the method of Runge et al. (2000).

The cells are visualized with Giemsa staining and the nucleus is stained with hematoxylin (Dako, Carpinteria, CA).

Establishment of primary human liver cell cultures:

The different culture conditions are tested to find the optimal combination of factors to grow human hepatocytes in culture based on the above mentioned conditions. The cells are plated at concentrations ranging from 5×10^4 – 1×10^6 /ml, on collagen or laminin coated plates, with serum free or serum containing medium. Various combinations of cytokines, such as HGF, EGF, IL-6, are evaluated and after 12 hours the medium is removed, the cells washed, and fresh medium added. Fresh medium is exchanged twice a week for the duration of the cultures. The technique adapted by Strom et al (1982) that involves a modification of the two-step collagenase perfusion technique is also used.

Expansion of hepatic or pancreatic cells:

The hepatic cells are expanded ex-vivo in the presence of LMW molecules such as Tepa ranging in concentrations from 5-25 μ M or in the presence of retinoic acid antagonists or CD38- inhibitory molecules. The expanded cells are selected for

subpopulations such as OC.3+ and will be induced to differentiate into pancreatic cell lines as detailed below. Similarly, the unexpanded cell population is selected first, then differentiated into pancreatic progenitor cells and only then ex-vivo expanded in presence of the above mentioned molecules.

Establishment of fetal hepatic cell cultures and their trans-differentiation into pancreatic-like cells

Cultures of sorted rat and human fetal hepatic stem cells, or a culture of pluripotent stem cells differentiated into hepatic-like cells, using the antigenic profiles such as c-met+, c-kit+, CD49f+, CD29+, CD49+, TER119+ are established and cultured as described by Yang et al (2002), in Iscove's modified DMEM serum-free medium containing LIF, IL-3, SCF, and flt-3 ligand, or in the presence of Gamida-Cell's (GC) expansion molecules such as Tepa (see expansion of hepatic cells). Differentiation is induced by switching the cells to RPMI medium with 10% fetal calf serum and high glucose (23mM).. Expression of islet beta-cell markers is determined by RT-PCR for PDX-1, PAX-4, PAX-6, insulin I, insulin II, glucagon and glucose transporter 2. Insulin, glucagon and pancreatic polypeptide hormone expression are also assessed by immunohistochemistry as described (Brill et. al., 1999; and Brill et al., 2002)

In vitro trans-differentiation of adult hepatic stem cells into pancreatic endocrine hormone-producing cells.

Hepatic progenitor cells are re-selected from the ex-vivo expanded cultures based on their size and expression of OC.3+. The re-selected cells are re-cultured under high-glucose environment followed by nicotinamide treatment.

Differentiation into pancreatic Islet-cells is examined. The gene expression profile of pancreatic specific genes such as PDX-1, PAX-4, PAX-6, Nkx2.2 and Nkx6.1, insulin I, insulin II, glucose transporter 2, and glucagons and the turn off of liver specific genes such as glucose 6-phosphate and α -fetoprotein is measured by use of RT-PCR and real time RT-PCR..

Alternatively hepatic cells derived from adult or fetal or differentiated pluripotent stem cell tissue are induced to differentiate into pancreatic insulin-

producing cells by genetic manipulations as described by Zalzman et al. (2003) or by Ferber et. Al., (2003) prior to, after or both prior to and after ex-vivo expansion.

Ex-vivo expanded bone marrow, peripheral blood and cord blood stem/progenitor (UCB) cells as a suitable source of functional active hepatic cells.

Freshly purified UCB mononuclear cells, or CD34+ or AC133+ populations are expanded ex-vivo by the method of Peled et al (2002). After 3 weeks incubation, the cells are washed with PBS and replated at a density of 4×10^4 /ml in hepatocyte medium culture.

Hepatic cells are derived from non-manipulated or from expanded UCB, PB or BM derived cells according to (Kakinuma et al., 2003). In brief, the cells are cultured with a combination of several growth/differentiation factors including FGF-1, FGF-2, LIF, SCF, HGF, and OSM or with a combination of 4 cytokines, SCF, TPO, FLt3 and IL-6, and a LMW modulator of self-renewal. The cells are plated on gelatin-coated tissue culture dishes. Determination of hepatocyte lineage generated from UCB, PB or BM cells is performed as detailed above. Alternatively, hepatic cells may be derived from hESC or other pluripotent stem cells cultured in the presence of n-butyrate, or n-butyrate-like compounds (Rambhatla et al., 2003)

Molecules that promote ex-vivo expansion

In all of the specified hepatocyte cell culture produced by induced-differentiation, the cells are expanded in the presence of retinoic acid inhibitors such as RA antagonist AGN 194310 (Allergen ,Irvine, CA) at concentrations ranging from 10^{-5} M to 10^{-9} M, or in the presence of Tepa (5-20 μ M), and CD38 inhibitors such as nicoinamide for time periods ranging from 1-3 weeks.

Characterization of hepatocytes

Hepatic cells are characterized by dye uptake, immunocytochemistry, FACS analysis, RT-PCR and by functional analysis as follows:

Cell staining: Cells are also stained with Giemsa staining using the manufactures protocol (Shandon, Pittsburg, PA). Cells are stained for 4 minutes at room temperature, washed in buffer solution for 4 minutes and washed 3-4 times with rinse solution.

Immunocytochemistry: Hepatocytes are also characterized by immunostaining of hepatic markers such as: albumin, α -fetoprotein, and cytokeratin 19 (cholangiocytes) using specific commercialized antibodies. The proteins were visualized by the Envision HRP-system (Dako) based on immunoperoxidase, diaminobenzidine tetrahydrochloride and H₂O₂ as the substrates following the manufacturer's instruction, or by fluorescence microscopy using secondary antibodies following manufacturing instructions (Jackson Laboratories). Cells are fixed in methanol at -20°C for 10 minutes, rinsed with PBS for 5 minutes and permeabilized with 0.1% triton (Sigma) in PBS for 5 minutes. The cells are washed with TBS for 5 minutes and incubated with 1% BSA in PBS for 10 minutes. Endogenous peroxidases are eliminated by incubating with peroxidase block (Envision) for 5 minutes. The cells are incubated for 30 minutes with antibodies against rabbit anti-mouse albumin (1:100) from Cappel (Aurora, Ohio); and against α -fetoprotein (1:25) AFP (H-140) from Santa Cruz Biotechnology, Inc, cytokeratin-19 1:10 (ICN, Irvine, CA). The samples were counter-stained with hematoxylin (Dako, Carpinteria, CA).

RNA and Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Total RNA is extracted using extraction by guanidine thiocyanate followed by phenol-chloroform and isopropanol precipitation. cDNA is synthesized from 1 μ g total RNA using random hexamer (pd(N)6) as a primer (Pharmacia Biotech; Uppsala, Sweden) and Moloney murine leukemia virus reverse transcriptase (GIBCO/BRL). cDNA samples are subjected to PCR amplification with DNA primers described in Table 1. For each gene, the DNA primers are derived from different exons to ensure that the PCR product represents the specific mRNA species and not genomic DNA. PCR is performed using the Clontech AdvantaqTM (Palo Alto, CA) RT-PCR kit and a two-step cycle at 68°C.

Quantitative analysis of specific genes expression by RT-PCR using β -Actin or 18S rRNA as housekeeping genes is performed. The expression level of hepatocytes-specific genes including albumin, AFP, α -trypsin, cytokeratin- 19, and glucose-6 phosphate, are assessed. The primers used are detailed in Table 1.

Table 1: Primers used for RT-PCR

Marker gene	Cell type specificity	5' primer	3' primer
Albumin	Mature hepatocytes	catgacaccatgcctgctgat	gtacacttccatgaagatcagag
α -fetoprotein	Young hepatocytes	actcaccccaaccttctgtc	ctctggtatcagccactgctg

Glucose-6 phosphate	hepatocytes	aacccattgtgaggccagagg	tactcattacactagttggtc
α -1 antitrypsin*	hepatocytes	tcgatcctaaggcacactgagg	cggcttgtaagactgttagc
Cytokeratin 19	Cholangiocyte	gtcctacagattgacaatgc	cacgctctggatctgtgacag
β -Actin**	housekeeping	atcatgtttagagaccttcaa	catctctgtcgaatcca

*Does not seem to be expressed when cells are plated on laminin

FACS: Multiparametric flow cytometric analysis (FACS) is used to identify sub-cellular populations of the adult expanded hepatic cells. FACS scatter analysis determines the less mature cells which are agranular from the more granular mature cells. Specific markers are used to study the liver progenitor cell populations comprising hepatic, hematopoietic, and mesenchymal progenitor cells. Staining of the expanded cells is performed with several fluoroprobe-labelled antibodies to specific cell surface markers that are shared with all of the progenitor cell populations as well as those that define unique lineages.

Cells are analyzed following trypsin digestion, centrifugation, and resuspension of cell pellets in phosphate-buffered saline (PBS). Prior to analysis, cells are filtered with a 40- μ M MESH filter (Falcon; Becton Dickinson) and kept on ice. Cells are washed with a PBS solution containing 1% BSA, and stained (at 4 °C for 30 minutes) with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated antibodies. The cells were then washed in the above buffer and analyzed by FACS calibur flow cytometer. Cells are passed at a rate of up to 1000 cells/second using a 488 nm argon laser beam as the light source for excitation. Emission of ten thousand cells is measured using logarithmic amplification, and analyzed using CellQuest software (BD). Background fluorescence is determined by staining cells with FITC- and PE-conjugated isotype control antibodies. Because of the false positive results frequently observed with FACS analysis of cultured cells, the content of progenitor cells is determined from a purified, re-selected fraction, using the chosen protocol. The re-selected cells are counted, giving absolute numbers of progenitor cells in the culture, and their antigenic expression is analyzed by flow cytometry. Percentages of early progenitor cell subsets are determined as well from the re-selected progenitor cell fraction. Cells are dually stained with CD38FITC and CD34PE for determination of CD34+CD38- cells; with CD34PE and a mixture of FITC-conjugated antibodies against CD38, CD33, CD14, CD15, CD3, CD61, CD19 for determination of CD34+Lin- cells (CD34PE, CD38 and

CD61FITC, DAKO (Glostrup, Denmark), CD33, 14, 15,3,19 FITC, BD (San Jose CA, USA), or other additional hematopoietic or hepatic markers.

Bioassays

Hepatocytes organic anions uptake: Cellular uptake of organic anions of hepatocytes is studied by Indocyanine green (ICG) dye uptake ICG (Sigma, Israel) dissolved in DMEM to a final concentration of 1 mg/ml (Yamada et al, 2002). Cells are washed twice with PBS and 400 μ l of the dye added. After 15 minutes incubation at 37 °C samples are rinsed 3 times with PBS.

Hepatic functional analysis. Albumin synthesis ability is analyzed by mRNA level using Northern analysis and by immunohistochemical staining using anti-albumin antibodies. Secretion of albumin to the medium is measured by ELISA. Detoxification functions of the hepatocytes is evaluated by the measurement of ureagenesis in the cell medium. The urea produced is determined by colorimetric assay for ureido compounds using antypirine and diacetylmonoxime. P-450 dependent ethoxycoumerine o-dealkylase activity is a measure of drug metabolizing activity by the expanded hepatocytes. Ethoxycoumerine o-dealkylase activity is monitored fluorometrically using multi-well plate scanner fluorometer.

In vivo hepatic models. The hepatic potential of the differentiated cells derived from the various sources described above by ex-vivo expansion with and without hepatic differentiation, and derived from either human liver tissue or from hematopoietic sources (BM, MPB, Cord blood) is evaluated by the capacity of the cells to engraft into injured liver in SCID mice (NOD/SCID or NOD/SCID/B2M) and/or immuno-deficient rats and to generate functional hepatocytes. Liver injury is induced by injection of CCl4 or by 0.4 mg/body of 2-acetylaminofluorene (2-AAF). Seven days after injection, mice are subjected to one-third partial hepatectomy. Evaluation of human cell engraftment and functionality is performed by the presence of human CD45+ cells expression of mRNA for human albumin, AFP+ and CK19+ and determination of human albumin in the serum of the mice by ELISA.

Diabetic animal models:

The potential of the expanded and non-expanded cells as well as the differentiated expanded and differentiated non-expanded cells to reverse

hyperglycemia is evaluated in 6-week old diabetic NOD-SCID mouse model as detailed by Zalzman et al. (2003).

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims. All publications, patents and patent applications mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention.

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Zalzman M, Gupta S, Giri RK, Berkovich I, Sappal BS, Karnieli O, Zern MA, Fleischer N, Efrat S. Reversal of hyperglycemia in mice by using human expandable

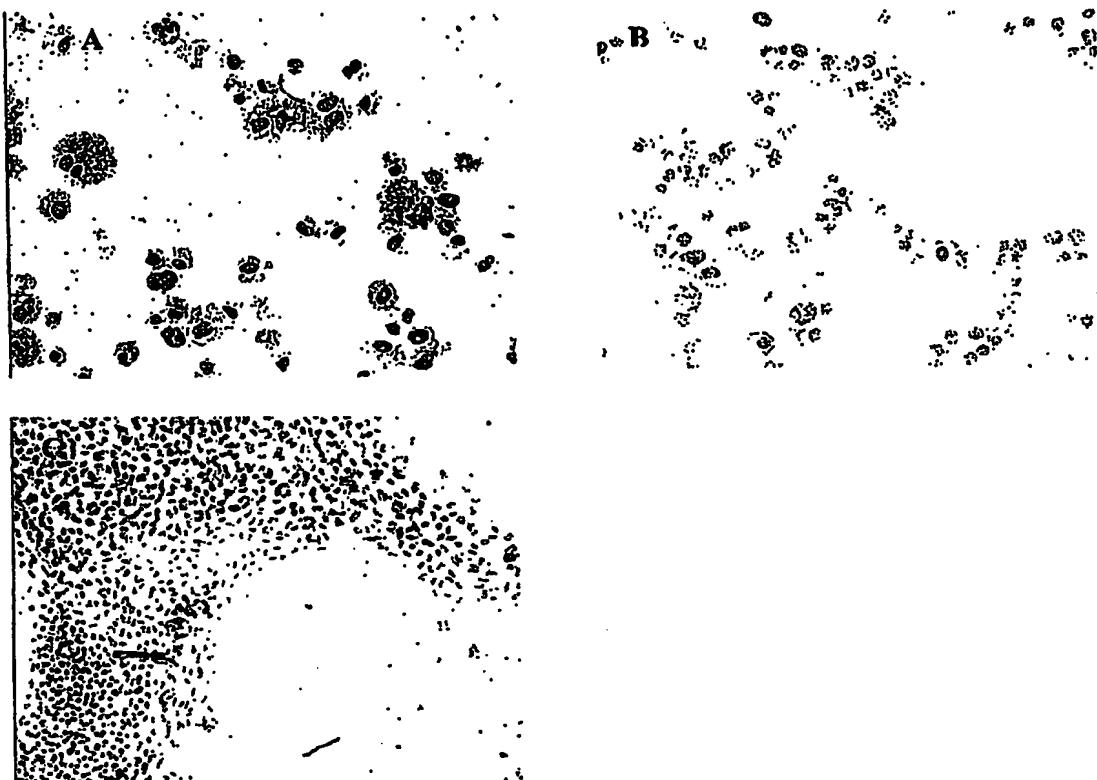
insulin-producing cells differentiated from fetal liver progenitor cells. Proc Natl Acad Sci U S A. 2003;100(12):7253-8.

Claims:

1. A method of ex vivo stem cell expansion of fetal/adult hepatic progenitor cells and hematopoietic stem cells essentially as described and exemplified herein.
2. Expanded cell population essentially as described and exemplified herein.
3. A method of treating liver and pancreatic diseases essentially as described and exemplified herein.
4. A method of treating liver and pancreatic diseases by implanting in a subject in need thereof the expanded cell population of claim 2.
5. A method of treating diabetes by implanting in a subject in need thereof the expanded cell population of claim 2.

Figure 1

Giemsa staining of hepatocytes harvested from 3 weeks old VLVC female mice. The cells were incubated with DMEM in the presence 0.05% collagenase for 30 minutes at 37 °C, grinded and pass through a 200 µm mesh. Cells are plated at a density of 4-x 10⁴/ml in F12 containing 15 mM Hepes, 0.1% glucose, 10 mM sodium bicarbonate, 100units/ml penicillin-streptomycin, glutamine, 0.5-units/ml insulin, 7.5m cg/ml hydrocortisone, and 10% fetal bovine serum, EGF (10ng/ml), and HGF (20ng/ml). Medium is changed after 12 hours, the cells washed twice with PBS and new medium added. Medium is changed twice a week.. (A) Cell grown in the absence of Tepa. (B), (C) Hepatocytes grown in the presence of 10 µM Tepa (20X).

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